EFFECT OF STORAGE TEMPERATURE ON THE GROWTH OF *LISTERIA MONOCYTOGENES* ON QUESO BLANCO SLICES*

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ABSTRACT

A five-strain cocktail of Listeria monocytogenes *(10^4 cfu/mL) was inoculated onto individual vacuum-packaged slices (ca. 50 g each) of a commercial, Hispanic-style cheese, that being Queso Blanco. Growth was determined at appropriate intervals during storage at 5, 10, 15, 20 and 25C. In general, as the incubation temperature increased, a shorter lag phase duration (LPD) and a faster growth rate (GR) were observed. The LPD values at 5, 10, 15, 20 and 25C were 65.3, 19.9, 2.1, 8.4 and 11.4 h, respectively. The GR values were 0.011, 0.036, 0.061, 0.090 and 0.099 log cfu/h at 5, 10, 15, 20 and 25C, respectively. There were no statistical differences in LPD at 10, 15, 20 and 25C. However, the LPD during growth at 5C was statistically ($P \leq 0.05$) longer than at all other temperatures. The GR values at 20 and 25C were not significantly different from each other, whereas the GR values at 5, 10 and 15C were significantly different from each other as well as from the GR at 20 and 25C ($P \leq 0.05$). The maximum population density (MPD) showed relatively little variation over the range of storage temperatures tested, with an average*

* Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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of 8.38 log cfu/g (SD = 0.33). The results of this study indicate that not even the lowest trial temperature of 5C prevented growth over time of the inoculated L. monocytogenes *on this sliced product, and that proper storage and handling procedures are required to prevent the bacterium from contaminating the product and/or to control its growth.*

INTRODUCTION

Listeria monocytogenes continues to be a significant threat to the safety of the food supply. Dairy products in general and Hispanic-style soft cheese in particular are known to harbor this pathogen. According to Altekruse *et al.* (1998), there were 32 cheese-associated outbreaks of human illness between 1973 and 1992 in the U.S.A., of which 11 were attributed to pathogen contamination either at the farm or during manufacturing/processing. These 11 outbreaks caused the vast majority of illnesses (1278 of 1700 reported) and hospitalizations (170 of 178 reported), and all 58 deaths among the 32 total outbreaks during this period. More important, of these 11 outbreaks that were attributed to contamination prior to distribution, five were associated with Hispanic-style soft cheese. One of these five outbreaks was due to *L. monocytogenes*. Ingestion of *L. monocytogenes* is particularly worrisome for susceptible populations, because for young, old, pregnant and immunocompromised individuals, the mortality rate is at least 20% (Mead *et al.* 1999). The 1985 California outbreak (142 cases and 48 deaths [Linnan *et al.* 1988]) attributed to the consumption of a Hispanic-style soft cheese that was prepared using unpasteurized milk still ranks as the largest listeriosis outbreak in North America. There was also a more recent outbreak of listeriosis (12 illnesses, 5 stillbirths [CDCP 2001]) in North Carolina in 2000 linked to a Hispanic-style cheese prepared from unpasteurized milk. Although there have been several studies (Genigeorgis *et al.* 1991b; Delgado da Silva *et al.* 1998; Saltijeral *et al.* 1999) conducted to determine the prevalence of *L. monocytogenes* in Hispanic-style cheese, there have been far fewer studies conducted to monitor its viability, to model its fate, and/or to control its occurrence.

Queso Blanco and Queso Fresco are the most recognized Hispanic-style soft cheese. Although they share similar characteristics, Queso Fresco is typically rennet set and finely milled making it more crumbly, whereas Queso Blanco is typically acid set and not finely milled, giving it a tougher texture (Van Hekken and Farkye 2003). Despite ongoing debate as to the definition and/or standard of identity for these types of cheese, there is little debate that they provide a favorable environment for the growth/survival of *L. monocytogenes* due to the relatively high moisture and low acid content of these varieties. Moreover, production is usually performed on a relatively small

scale, typically by local producers, and it involves considerable hand manipulation, all of which contribute to a higher probability of contamination by *L. monocytogenes*. Such attributes are reflected in a risk assessment by federal agencies in the U.S.A. wherein fresh soft cheese along with paté and meat spreads were ranked as the top two selected categories of ready-to-eat foods for listeriosis on a per serving basis among 23 food categories surveyed (www.foodsafety.gov/~dms/lmr2-toc.html). These data, coupled with Census Bureau projections that the Hispanic population in the U.S.A. will continue to grow from about 35 million in 2000 to about 55 million by 2020 (Hollingsworth 2003), would suggest that both the consumption of Hispanic-style cheese and, in turn, the risk of listeriosis will continue to increase. As such, the primary objective of the present study was to model the growth of *L. monocytogenes* in Queso Blanco.

MATERIALS AND METHODS

The five isolates of *L. monocytogenes* used in this study (Table 1) were confirmed, cultured and maintained as described previously (Porto *et al.* 2002). For each experiment, a single colony of each isolate was separately transferred into 50 mL of brain–heart infusion (BHI; Difco Laboratories, Detroit, MI) broth and incubated at 37C for 24 h with shaking. The resulting stationary-phase cells of each of the five cultures were diluted separately in 0.1% peptone water (Bacto Peptone; Difco) to achieve a final concentration of

TABLE 1.
STRAINS OF *LISTERIA MONOCYTOGENES* USED IN THIS STUDY

Strain number	Other Designation	Source	Serotype	Reference/Source
MFS 53	F2365	Hispanic-style cheese isolate, 1985, California outbreak	4b	Linnan *et al.* 1988; Nelson *et al.* 2004
MFS 1365	JBL2365	Chocolate milk isolate, 1994, Illinois/Wisconsin outbreak	1/2b	Proctor *et al.* 1995; Dalton *et al.* 1997
MFS 104	Scott A	Clinical isolate, 1983, Massachusetts pasteurized milk outbreak	4b	Fleming *et al.* 1985
MFS 1363	L628	Butter isolate, 1999, Finland outbreak	3a	Lyytikainen *et al.* 2000
MFS 1394	Mex-5	Hispanic-style cheese isolate, 2002, Sonora, Mexico	4b	Martha Diaz, Centro de Investigacion en Alimentacion y Desarrollo, Hermsillo, Sonora, Mexico.

approximately 10^4 cfu/mL, and a 10-mL portion of each of the five isolates was combined to generate the cocktail that was used to inoculate the Queso Blanco.

A single batch of Queso Blanco, a semi-soft white cheese composed of pasteurized milk, salt, enzymes and commercial cheese cultures (lactic starter, soy protein), but without any additional cultured milk additives, was provided fresh in either 5- or 1-lb blocks by a cooperating commercial producer. A 2-lb frozen sample of Queso Blanco was sent to a commercial facility (Southern Testing & Research Laboratories, Wilson, NC) for chemical analyses using the methods described and approved by the Association of Official Analytical Chemists (1998). The remainder of the cheese was sliced (ca. 50 g each, ca. 5 cm × 5 cm × 5 mm per slice) using a Globe 3500 Manual Stainless Steel Slicer (Globe Food Equipment Co., Dayton, OH), and then repackaged (one slice per bag) into 400-mL-capacity sterile filter bags (polyester/polyethylene, 70-μm thickness, 19 × 30 cm; Spiral Biotech, Norwood, MA). The packages were vacuum-sealed to 950 m Bar using a Multivac A300/16 vacuum-packaging unit (Sepp Haggemüller KG, Wolfertschwenden, Germany). The sliced cheese was immediately frozen at −20C and held for up to 60 days. A portion of the frozen samples was irradiated for a companion study (manuscript in preparation). In control experiments, we compared the growth of the five-strain cocktail of *L. monocytogenes* on fresh and frozen vacuum-packaged cheese slices at 10 and 25C, and found no significant differences in growth rate (GR), lag phase duration (LPD) or maximum population density (MPD) (data not shown). There were also no appreciable changes in pH or moisture content following freezing and/or irradiation.

One day prior to experimentation, cheese samples were removed from −20-C storage and thawed at 4C. The packages were opened with ethanol-sterilized scissors, inoculated with either 200 μL of the five-strain *L. monocytogenes* cocktail or 200 μL of sterile 0.1% peptone water (noninoculated control), and vacuum sealed. The samples were incubated at 5, 10, 15, 20 or 25C and at the appropriate sample points, three (5 and 10C) or two (15, 20 and 25C) packages were reopened using ethanol-sterilized scissors, combined with 10-mL sterile 0.1% peptone water, and stomached for 60 s (Bagmixer 400; Interscience, St Nom, France). A 50-μL portion of the recovered homogenized mixture was spread plated, with and without prior dilution in 0.1% peptone water, onto duplicate BHI agar plates for enumeration of total bacteria (TB), modified Oxford agar (Cook 1999) for enumeration of *L. monocytogenes*, and/or Lactobacilli MRS agar (Difco) for enumeration of total lactic acid bacteria (LAB). Queso Blanco samples inoculated with *L. monocytogenes* were analyzed for TB and *L. monocytogenes* at every sampling point for each temperature tested. Counts of LAB in the inoculated samples were determined

at time zero, once during the exponential growth phase and once during the stationary growth phase. Control samples inoculated with 0.1% peptone water were sampled for TB, *L. monocytogenes* and LAB at time zero, once during the exponential growth phase and once during the stationary growth phase. All plates were incubated aerobically at 37C for 24 to 48 h and colonies were counted manually. *Listeria* colonies were identified and confirmed as described previously (Porto *et al.* 2002). Bacterial numbers were expressed as log cfu/g.

The DMFit software (courtesy of J. Baranyi, Institute of Food Research, Norwich, U.K.) was used to fit the D-model (Baranyi *et al.* 1993; Baranyi and Roberts 1994) to the primary time-dependent data. Secondary models were developed using TableCurve 2D (SPSS Inc., Chicago, IL) software containing preset and customized algorithms. Means separations were performed using the pair-wise least significance difference method. The level of significance for all comparisons was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Listeria monocytogenes is frequently associated with Hispanic-style cheese, including Queso Blanco, and attendant listeriosis episodes. In addition, Queso Blanco is quite often produced in private homes using raw milk, making food safety regulations difficult to enforce. Thus, culturally appropriate education efforts should be expanded to avoid such problems in the future. The use of raw milk is a risk for any cheese, since surveys have identified raw milk as a significant source of *L. monocytogenes*, with 1.6 to 7.0% of such samples testing positive in the United States (Ryser and Marth 1999). Moreover, cheese produced from pasteurized milk may still represent a risk for listeriosis given that contamination may also occur during processing and/or postprocessing.

Although several studies have established that a variety of soft cheese supports the growth of *L. monocytogenes*, these data cannot be used to predict the probability of *L. monocytogenes* growth in Queso Blanco (Glass *et al.* 1995; Ramsaran *et al.* 1998; Solano-López and Hernández-Sánchez 2000). In this study, we developed a model for the growth of *L. monocytogenes* in Queso Blanco at 5, 10, 15, 20 and 25C. At each of the storage temperatures tested, we observed lag, growth and stationary phases for a five-strain cocktail of *L. monocytogenes* (Fig. 1). The D-model described by Baranyi and coworkers (Baranyi *et al.* 1993; Baranyi and Roberts 1994) was used to estimate parameters for LPD, GR and MPD. The GR values at 5, 10, 15, 20 and 25C were 0.011, 0.036, 0.061, 0.090 and 0.099 log cfu/h, respectively (Table 2). The GR values at 20 and 25C were not significantly different from each other, whereas

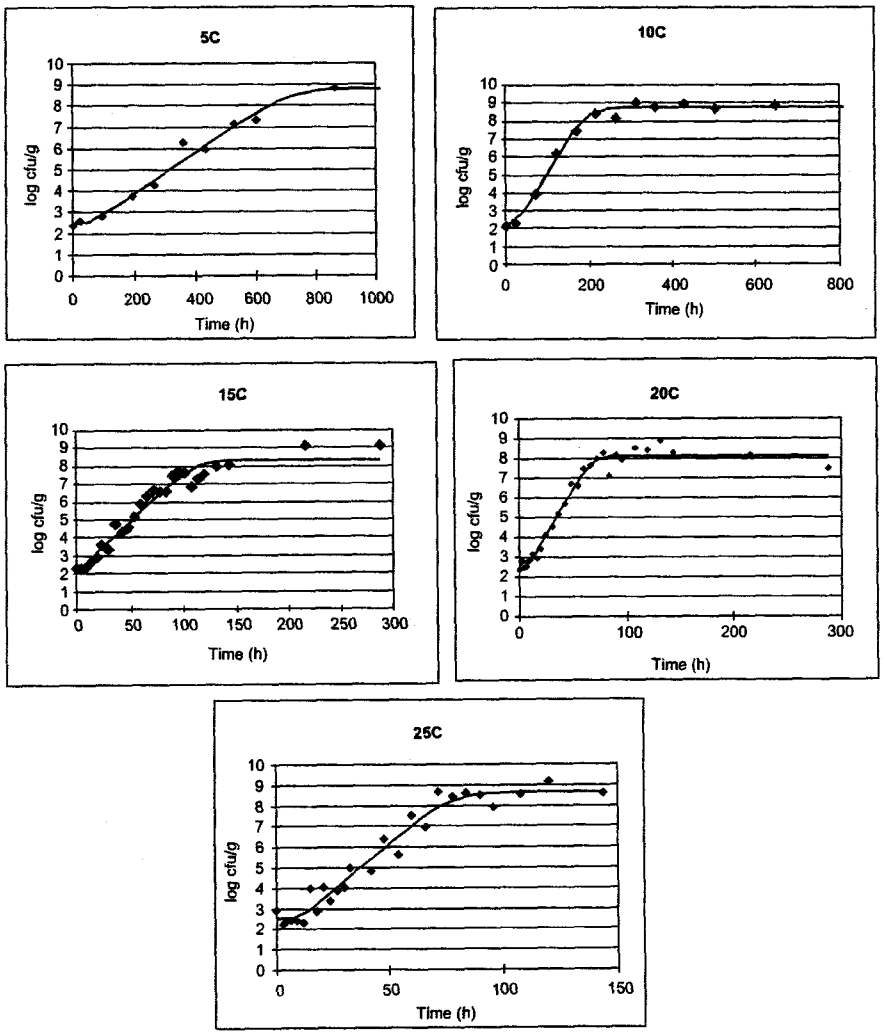

FIG. 1. KINETIC DATA (◆) AND PRIMARY CURVE FITS (SOLID LINE) FOR SELECTED EXPERIMENTS ON THE GROWTH OF *LISTERIA MONOCYTOGENES* IN QUESO BLANCO AT 5, 10, 15, 20 AND 25C. (◆) REPRESENTS OBSERVED *L. MONOCYTOGENES* COUNTS CALCULATED FROM DUPLICATE PLATES FROM EACH OF THREE INDIVIDUAL SAMPLES FOR 5 AND 10C AND TWO INDIVIDUAL SAMPLES FOR 15, 20 AND 25C FOR A SINGLE TRIAL

TABLE 2.
LAG PHASE DURATION (LPD), GROWTH RATE (GR) AND MAXIMUM POPULATION DENSITY (MPD) OF *LISTERIA MONOCYTOGENES* IN QUESO BLANCO STORED AT 5, 10, 15, 20 AND 25C*

Temperature (C)	LPD (h)†	GR (log cfu/h)‡	MPD (log cfu)§
5	65.34 ± 17.11^{a}	0.011 ± 0.001^{d}	7.99 ± 0.74^{a}
10	19.93 ± 15.62^{b}	0.036 ± 0.005^{c}	8.80 ± 0.05^{a}
15	2.14 ± 1.18^{b}	0.061 ± 0.005^{b}	8.32 ± 0.05^{a}
20	8.36 ± 0.63^{b}	0.090 ± 0.007^{a}	8.17 ± 0.04^{a}
25	11.41 ± 2.04^{b}	0.099 ± 0.011^{a}	8.64 ± 0.01^{a}

* Values represent the mean ± SD of duplicate plates of each dilution from each of three (5 and 10C) or two (15, 20 and 25C) samples from a single trial.
† LPD values with the same letter are not significantly different.
‡ GR values with the same letter are not significantly different.
§ MPD values with the same letter are not significantly different.

the GR values at 5, 10 and 15C were significantly different from each other as well as from the GR values at 20 and 25C ($P \leq 0.05$). In comparison with GR predicted using a pure culture, in a broth-based model for *L. monocytogenes* strain Scott A under anaerobic conditions (Buchanan *et al.* 1989; Buchanan and Phillips 1990), the GR values in Queso Blanco were approximately five to seven times lower. Specifically, the broth model predictions at 5, 10, 15, 20 and 25C were 0.083, 0.17, 0.31, 0.49 and 0.69 log cfu/h, respectively. The distribution of GR could be described with the Ratkowsky square root model (Eq. 1; Ratkowsky *et al.* 1983).

$$GR = (b \times [temperature - T_{min}])^2 \quad (1)$$

where $b = 0.009$, $T_{min} = -10.44$, and $R^2 = 0.94$.

Bacterial LPD is a reflection of the time that cells require to adjust physiologically to an environment prior to cell replication (Baranyi *et al.* 1993; Baranyi and Roberts 1994). Generally, LPD decreases with increasing temperature, and is affected by the previous environment to which the cells are exposed and by inherent physiological variations among bacterial cells within a single population (Metris *et al.* 2003; Baranyi and Pin 2004). The LPD values at 5, 10, 15, 20 and 25C were 65.3, 19.9, 2.1, 8.4 and 11.4 h, respectively (Table 2). The LPD in Queso Blanco stored at 5C was significantly ($P \leq 0.05$) longer than the LPD noted for each of the other storage temperatures. This distribution of LPD could be described with an exponential secondary model (Eq. 2). Similar to other reports, our results showed higher variation for LPD (Hwang and Tamplin 2005; Tamplin *et al.* 2005).

Specifically, the average ratio of the SD to the means of the replicate treatments for the five temperature treatments was 0.099 for GR and 0.370 for LPD.

$$LPD = a + b^{(-temperature/c)} \quad (2)$$

where $a = 5.28$, $b = 881.11$, $c = 9.93$, $R^2 = 0.97$.

The MPD ranged from 7.99 to 8.80 log cfu/g, with an average of 8.38 log cfu/g (SD = 0.33). There were no significant differences in MPD among the five storage temperatures tested (Table 2). These findings are consistent with results reported by other researchers (Genigeorgis *et al.* 1991b; Back *et al.* 1993; Papageorgiou *et al.* 1996; Mendoza-Yepes *et al.* 1999; Leuschner and Boughtflower 2002) who demonstrated the growth and/or survival of *L. monocytogenes* in soft cheese over a broad range of storage temperatures.

Regardless of the storage temperatures tested, TB populations (8.59 to 9.77 log cfu/g) showed a similar growth rate to the five-strain cocktail of *L. monocytogenes* in Queso Blanco. Moreover, *L. monocytogenes* was not detected ($\leq$2 log cfu/g) in the uninoculated control samples by direct plating on day zero or at selected sampling points during the log or stationary phases. In related experiments (data not shown), we also monitored the fate of *L. monocytogenes* in Queso Blanco sterilized by irradiation (42 kGy). Our preliminary results indicated that, in nonirradiated samples, the indigenous cheese microflora and the inoculated *L. monocytogenes* grew at a similar rate. However, in the irradiated samples, the inoculated *L. monocytogenes*, without competition from the indigenous microflora, showed a smaller cumulative change in growth (i.e., log cfu final – log cfu initial) at each time point compared with the change in growth of *L. monocytogenes* in nonirradiated Queso Blanco. Further work is in progress to verify these findings.

In this study, we developed a model for the growth of a five-strain mixture of *L. monocytogenes* in Queso Blanco at 5, 10, 15, 20 and 25C for up to 1032 h of storage. However, the model developed for these growth parameters should be validated in other soft cheese. Formulations and manufacturing practices for Mexican-style cheese present a significant risk for product contamination and subsequent growth by *L. monocytogenes* (Glass *et al.* 1995, 1998). Proximate composition analyses of the commercial cheese used in this study (Table 3) indicate a composition typical for Queso Blanco (http://www.ams.usda.gov/fqa/proposed%20cids/aa20347.htm). The relatively low acid (pH 6.8) and high moisture content (48%) of the Queso Blanco used in this study provided a favorable environment for the growth of *L. monocytogenes* at the various temperatures tested. Although the pH of the commercial Queso Blanco we used in this study was somewhat higher than what is typical

TABLE 3.
PROXIMATE COMPOSITION OF QUESO BLANCO*

Analyses	Queso Blanco composition	Commercial Queso Blanco Description†
Acidity as lactic acid (g/100 g)	<0.01	–
pH	6.80	5.25–5.9
Salt (g/100)	2.32	1.8–3.0
Moisture (g/100 g)	48.8	45.0–55.0
Ash (g/100 g)	4.71	–
Fat (g/100 g)	18.2	18.5–25.0
Saturated fat (g/100 g)	11.2	–
Protein (g/100 g)	19.6	20.0–22.0
Water activity (A_w)	0.971	–
Carbohydrates (g/100 g)	8.09	–

* Values represent the results from single analyses performed on a single sample taken from a single batch collected on sampling day zero.

† Commercial item description according to the Agricultural Marketing Service of the U.S. Department of Agriculture (www.ams.usda.gov/fqa/proposed%20cids/aa20347.htm).

(Table 3), similar values (pH 6.2 to 6.7) have been reported for seven other commercial soft Hispanic-style cheeses (Genigeorgis *et al.* 1991b). Regardless, we evaluated retail Queso Blanco with characteristics typically attributed to this type of Hispanic-style cheese. These findings are consistent with the findings of Genigeorgis *et al.* (1991a) who reported that *L. monocytogenes* survived and grew in a variety of Hispanic-style cheese stored at 4 to 30C.

The reported prevalence of *L. monocytogenes* in soft cheese was <1 to 6%, with pathogen levels ranging from 1 to 5 log cfu/g (Loncarevic *et al.* 1995; Papageorgiou *et al.* 1998). Our results indicate that *L. monocytogenes* inoculated into Queso Blanco at ≤3 log cfu/g will increase to ≥6 log cfu/g within 17 days at 5C. At a less desirable temperature such as 10C, pathogen numbers will reach ≥8 log cfu/g within 8 days. According to the Agricultural Marketing Service (AMS) of the U.S. Department of Agriculture (USDA; www.ams.usda.gov/fqa/ciddair.htm), Queso Blanco should have a shelf life of 90 days when stored at 0.6 to 3.3C. Our findings indicate that during the storage of Queso Blanco at 5C, *L. monocytogenes* will reach approximately 8 log cfu/g (MPD) within ≤35 days. These findings suggest that *L. monocytogenes* can grow to its maximum density in Queso Blanco well within the expected product shelf life during storage at refrigeration temperatures.

The information generated in this study may be used in food safety assessments to predict the growth of *L. monocytogenes* in high moisture and low acid soft cheese during storage at 5 to 25C. These data also demonstrate that proper storage temperatures alone are not sufficient to inhibit or slow the growth of *L. monocytogenes* in such products. Further studies are warranted to

identify and optimize formulations and/or interventions to better control *L. monocytogenes* should it become associated with Queso Blanco at any time prior to consumption.

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